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Review

Structure and organization of chromatin fiber in the nucleus

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ABSTRACT

Eukaryotic genomes are organized hierarchically into chromatin structures by histones. Despite extensive research for over 30 years, not only the fundamental structure of the 30-nm chromatin fiber is being debated, but the actual existence of such fiber remains hotly contested. In this review, we focus on the most recent progress in elucidating the structure of the 30-nm fiber upon in vitro reconstitution, and its possible organization inside the nucleus. In addition, we discuss the roles of linker histone H1 as well as the importance of specific nucleosome-nucleosome interactions in the formation of the 30-nm fiber. Finally, we discuss the involvement of structural variations and epigenetic mechanisms available for the regulation of this chromatin form.

1. Introduction

In eukaryotic cells, the genomic DNA must be tightly packaged into chromatin to fit inside a nucleus that has a diameter of only a few microns. During the last three decades, the structure of chromatin has been extensively studied. Early studies had already revealed that the basic repeating structural unit of chromatin is the nucleosome, and it is now well established that it is comprised of the core particle and linker DNA [1]. The nucleosome core particle (NCP) consists of 147 base pairs (bp) of DNA wrapped around an octamer of histones, with two copies of each H2A, H2B, H3 and H4, and about 1.7 superhelical turns arranged in a left-handed manner [2]. The nucleosome cores are connected by linker DNA, which typically ranges from 10 to 90 bp in length, to form a "beads-on-a-string" nucleosomal array with a diameter of 11 nm. The nucleosomal array represents the first level of DNA compaction [3]. Linker histones (H1 and H5) bind to the DNA linker regions in close proximity to the sites of DNA entry and exit to the NCP, and organize the nucleosomal arrays into a more condensed 30-nm chromatin fiber, regarded as the second level of DNA compaction [4,5]. The structure of the NCP has been determined by X-ray crystallography at 1.9–2.8 Å resolution [2,6]. However, despite considerable efforts during the last three decades, the structure of the 30-nm fibers, together with the role of linker histones in its formation, still remains to be resolved [3,7]. Whilst biochemical and structural data suggest that the folding of nucleosome arrays is mainly driven by nucleosome-nucleosome interactions, the precise path of the linker DNA within the fiber is still incompletely understood. Since a high-resolution crystal structure of the chromatosome (NCP with linker DNA and linker histone) is still lacking, the precise location of the linker histone in the chromatosome remains under debate [8,9].

The organization of genomic DNA into a chromatin structure plays a critical role in the regulation of gene transcription and all other biological processes involving DNA, such as DNA replication, repair and recombination. The 30-nm fiber has been shown to be the first level of transcriptionally dormant chromatin by in vitro experiments [10], thus one critical function of the 30-nm chromatin fiber in such processes might be to regulate the accessibility of trans-acting factors via dynamic transitions between the more compact 30-nm chromatin fiber and more accessible nucleosomal arrays [3,10]. Understanding the structure of the 30-nm chromatin fiber is of great importance to illuminate in detail the functions and molecular mechanisms of chromatin dynamics in epigenetic regulation during gene expression and other DNA-related processes [3]. However, essential details regarding the formation and regulation of the 30-nm chromatin fiber are less well understood. In this review, we focus on the most recent progress in elucidating the structure of the 30-nm chromatin fiber reconstituted in vitro as well as its organization within the nucleus. In addition, we discuss the roles of linker histone H1 and nucleosome-nucleosome interactions in the formation of 30-nm chromatin. Lastly, we will summarize our current knowledge concerning the structural variations

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and dynamics of the 30-nm chromatin fiber in the epigenetic regulation of eukaryotic gene expression.

2. Structure of the 30-nm chromatin fiber: solenoid vs. zig–zag

Under physiological conditions, nucleosomal arrays have an inherent propensity to coil into condensed chromatin fibers with a diameter of ~30 nm. Based on the early studies of native chromatin in nuclei or isolated from nuclei by various biochemical and biophysical studies, a number of models, including the solenoid [11,12], twisted-ribbon [13,14], cross-linker [15,16], and superbead [17] models, had initially been proposed for the three-dimensional organization of nucleosomes into 30-nm chromatin fibers. The study of chromatin isolated from nuclei has advantages in that it presumably represents the "native" state. However, heterogeneous properties of nucleosomes in native chromatin with different DNA sequences, variable linker DNA lengths and different histone modifications/compositions make it difficult to define the detailed structure of chromatin fibers and to trace the paths of nucleosomal arrays. In an attempt to reduce the effect of these variables, scientists have developed a well-defined in vitro reconstituted nucleosomal array system that incorporates a strong nucleosome positioning sequence into the DNA. The positioning sequence is a 208-bp DNA fragment isolated from a *Lytechinus variegatus* 5S rRNA gene [18]. Using this system, numerous studies have shown that the reconstituted nucleosome arrays in the absence of linker histones can reversibly fold into secondary chromatin structures that resemble structures formed by native chromatin that lacks H1 [19]. Moreover, nucleosomal arrays reconstituted with H1 behave similarly to native chromatin, folding in the presence of monovalent or multivalent ions in vitro [20]. Thus, the reconstituted system reflects the ability of native chromatin to form primary, secondary and tertiary chromatin structures.

Recent development of the synthetic 601 family of nucleosome positioning sequences [21] has led to the construction of arrays with extremely well-defined nucleosome positions [22]. This technology has greatly improved the reproducibility and uniformity for structural analysis, and allowed for a dissection of the contribution of different nucleosome repeat lengths (NRLs). Based on the measurements of these reconstituted chromatin fibers in vitro by electron microscopy and analytical ultracentrifugation, two basic classes of structural models, namely the one-start solenoid model and the two-start cross-linker model, have been proposed. In these models, nucleosomes are either arranged linearly in a one-start solenoid-type helix with a bent linker DNA, or they zig–zag back and forth in a two-start stack of nucleosomes connected by a relatively straight linker DNA [22,23]. Dorigo et al. studied the reconstituted oligonucleosome arrays containing recombinant core histones using NRLs of 167, 177 and 208 bp, in either presence or absence of linker histone H1. EM photographs of these reconstitutes showed two-start flat ribbons with about 5 nucleosomes per 11 nm in length [22], rather than the helical arrangement with about 6–7 nucleosomes per 11 in nm length, as observed previously in isolated native chromatin [11]. In a subsequent landmark study, Schalch et al. solved the crystal structure of tetranucleosome with NRL of 167 bp in the absence of linker histones to a resolution of 9 Å, and revealed a structure with nucleosomes stacked perpendicularly to its axis [24]. Although the resolution was relatively low, the overall structure clearly showed two rows of two nucleosome stacks with the three-linker DNA segments criss-crossing between them, thus supporting the zig–zag (cross-linker) model of the 30-nm fiber. Importantly, this zig–zag conformation is in agreement with the in vitro crosslinking studies performed in solution with longer nucleosomal arrays (12 nucleosome repeats) [22,25] and in vivo analysis of chromatin fragmentation patterns generated by ionizing radiation [26]. Their proposed idealized model is a twisted ribbon with a diameter of about 25 nm and a compaction density of 5–6 nucleosomes per 11 nm.

In another recent study, Rhodes and colleagues analyzed the structures of long and regular chromatin fibers reconstituted with discrete NRL (from 177 to 237 bp, at 10 bp-intervals) by EM and cryo-EM in the presence of linker histones [23]. Although the detailed structure could not be resolved, the dimensions measured allowed the author to propose a one-start interdigitated solenoid structure with a fiber diameter of 30 nm [23]. Rhodes and colleagues found that both linker histone and NRL determine the structure of chromatin fiber [27]. In the absence of linker histone, the 167-bp NRL array displayed a highly ordered "ladder"-like structure consisting of stacked nucleosomes in two-start helix arrangement, which is reminiscent of that previously observed by Richmond and colleagues of the 167-bp NRL nucleosome array [22]. In addition, Robinson et al. also showed that their reconstitutes comprise two additional classes of structures; one with repeat lengths of up to 207 bp and a diameter of 33 nm, the other with repeat lengths of 217, 227 and 237 bp and a diameter of 44 nm [23].

Thus, despite three decades of intense research, the precise structure of the 30-nm chromatin fiber remains elusive, with the consensus viewpoint being that there is a see-sawing back and forth between a one-start solenoid and a two-start zig–zag architecture. In the above studies, the arrangements of nucleosomes and linker DNA within the 30-nm chromatin fiber have not been resolved. Most recently, we determined the 3D cryo-EM structure of the 30-nm chromatin fiber at a resolution of about 11 Å. The fibers were reconstituted in vitro from arrays of 12 nucleosomes with linker histone H1 (Fig. 1, panel A). Our structures of 30-nm fibers provide the most detailed view of the intrinsic structure of a linker histone-containing chromatin fiber, and our structures clearly reveal a histone H1-dependent, left-handed twist of the repeating tetranucleosomal structural units [28] (Fig. 1, panel B and C). The structures constitute the largest fragments of chromatin fibers revealed at this resolution, allowing a clear definition of the spatial location of all individual nucleosomes and tracing the path of linker DNA (Fig. 1, panel A). Different NRLs (177-bp or 187-bp 601 DNA sequence) did not affect the overall architecture of 30-nm fibers but changed the fiber dimension, which is consistent with the fundamental prediction of a basic zig–zag two-start helix model [14,16,22,24]. Interestingly, a tetranucleosomal repetitive unit was observed in the cryo-EM structures of the 30-nm chromatin fiber (Fig. 1, panel B and C; Fig. 2, panel A and B). The architecture of tetranucleosomal unit appears very similar to the resolved X-ray structure of a tetranucleosome with the 167-bp NRL in the absence of linker histone [24] (Fig. 2, panel C and D). The results indicate that the presence of H1 and the length change of linker DNA by 10 bp do not affect the interactions within the nucleosome stack, but affects the separation and rotation between the two stacks. In addition, the 3D cryo-EM structure also shows that the packing density of the left-handed helical 30-nm fiber is about 6.1–6.4 nucleosomes per 11-nm turn, which agrees well with many other studies [5], but which is lower than the density of the more compacted fibers (10–12 nucleosomes per 11 nm) assembled under conditions that favor more charge neutralization [23]. In principle, a higher packing density could be accommodated by a concertina-like motion that would reduce the vertical separation between successive turns of the fiber [29]. Although our 3D cryo-EM structures for the reconstituted 30 nm chromatin fiber with NRLs of 177- and 187-bp show a left-handed twist of the repeating tetra-nucleosomal structural units with a two-start "Zig–Zag" configuration, other forms of chromatin structures may exist in different conditions, for example, the one-start "Solenoid" structure in the presence of H5 and magnesium with longer NRLs as discussed above [23].

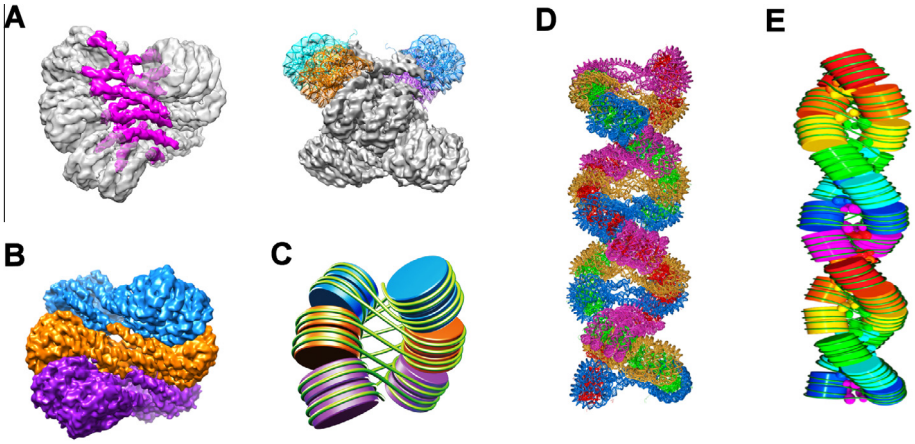

Fig. 1. 3D structure of 30-nm chromatin fiber. (A) The overall structure of 30-nm chromatin fibers reconstituted on 12 × 187 bp DNA arrays viewed from two angles. (B) The three tetranucleosomal structural units of the 30-nm chromatin fibers reconstituted on 12 × 187 bp DNA arrays are highlighted in different colors as shown in A. (C) A schematic representation of the cryo-EM structure of a 30-nm chromatin fiber as shown in B. (D) A pseudo-atomic model built from the cryo-EM structure of the dodeca-nucleosomal 30-nm fiber. (E) A schematic representation of the cryo-EM structure of the 30-nm chromatin fibers as shown in D. Adapted from [28].

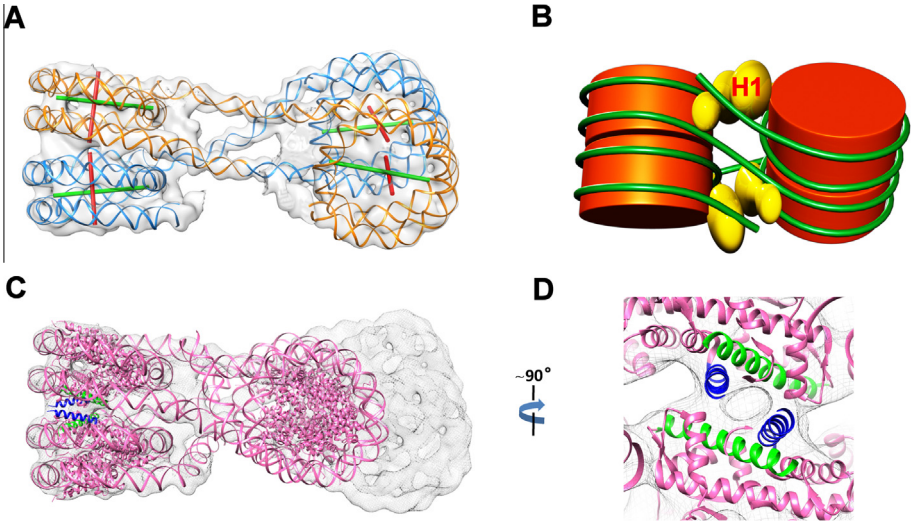

Fig. 2. Arrangement of nucleosomes within a tetranucleosomal unit. (A) The segmented density map (grey) for the tetranucleosomal unit in the 30-nm chromatin fibers reconstituted on 12 × 187 bp DNA arrays shown with the atomic structure of DNA from a docked mono-nucleosome crystal structure (PDB ID: 1AOI). Different axes are highlighted by different colors. (B) A schematic representation of the cryo-EM structure for the tetranucleosomal unit as shown in A. (C) A comparison of the 3D cryo-EM map (grey) with the X-ray structure (PDB ID: 1ZBB, pink) of the tetranucleosome [24]. (D) The strong density where the adjacent H2A–H2B dimer meets is magnified and highlighted in the interface between the nucleosome cores within each stack. Adapted from [28].

3. The role of H1 in setting up the 30-nm chromatin fiber structure

In most eukaryotes, a family of histone proteins named linker histones (also referred to as H1 and H5), can be found in highly condensed chromatin, with a stoichiometry of approximately 1:1 of linker histone:nucleosome. Linker histones are typically ~200 amino acids (aa) in length, and are of tripartite organization. Here, a ~70–80 aa structured globular winged helix domain (WHD) [30] is flanked by a short, disordered N- terminal tail and a ~100 aa long, apparently unstructured C-terminal tails that is highly enriched in lysines [31,32]. Considerable evidence has shown that the linker histones are located at the entry/exit point of the linker DNA in the chromatosome [33,34]. Also, the globular domain of H1/H5 (gH1/gH5) was shown to specifically bind to the cruciform-like structural organization of DNA at the entry and exit site protruding from the nucleosome [33–35]. In addition, it has been shown that the correct positioning of the C-terminal tail of H1 is required for both its binding to chromatin in vivo [36], as well as for the formation of a stem structure of linker DNA in vitro [8,35,37]. However, the N-terminal region of H1 has been shown not necessary for nucleosome binding; furthermore, the precise binding sites of the N-terminal region are not yet clearly established [33,35].

Histone H1 plays an important role in the formation and stabilization of the 30-nm chromatin fiber [7]. However, both the exact position of the linker histone H1/H5 on nucleosome within 30-nm fiber and its precise function during the formation of 30-nm fiber remains to be determined structurally. Binding of a single linker histone to nucleosomes protects an additional 20 bp of linker DNA from micrococcal nuclease digestion [38]. The putative location of H1 (or GH1) in the chromatosome have been extensively discussed elsewhere [39–41]. Two classical models, one symmetrical and one asymmetrical binding model, have been proposed for the binding of H1 to the nucleosome, as well as for the location of gH1/gH5 in the chromatosome. Based on micrococcal nuclease

digestion and DNase I footprinting experiments, a symmetrical model was proposed, in which gH1/gH5 is centrally located on the dyad-axis of the nucleosome, probably interacting with both entry-exit linker DNAs at the dyad, thereby protecting them symmetrically (about 10 bp at each end) from further digestion [42,43]. However, more recent studies on a sea urchin 5S rDNA sequence [44,45] as well as on the thyroid hormone response element [46] have shown that chromatosome protection was asymmetric, with the additional 20 bp all at one end of the core particle being protected.

Interestingly, in our recently published cryo-EM structure of the dodeca-nucleosomal 30-nm fiber, the twelve H1 molecules are clearly visible, exhibiting a proper 1:1 stoichiometric association with the nucleosome cores (Fig. 3, panel A). The globular domain of histone H1 is shown to be internally located, relative to the axis of the 30-nm chromatin fiber (Fig. 3, panel A), which is consistent with previous observations [47]. In addition, our cryo-EM structure visualized for the first time the location of H1, and thus elucidated its precise role in the formation of the 30-nm fiber. An apparent off-axis asymmetric binding of the globular domain of H1 in the chromatosome can be clearly seen in the cryo-EM structure (Fig. 3, panel B). This asymmetric location of H1 in the chromatosome thus confers polarity on the symmetric core nucleosome. In such a way, successive nucleosomes in the same stack have opposite polarities within the tetranucleosomal unit, which allows the self-association of the globular domain of the linker histone H1 between tetranucleosomal units in the fiber (Fig. 3, panel A; Fig. 4, panel A). H1–H1 self-associations had been reported earlier to play an important role in the organization and stabilization of the 30-nm chromatin fiber [48,49]. The self-association of the linker histone H1 between tetranucleosomal units thereby can stabilize coherent stacking in the tetranucleosome unit, and thus impart an additional twist between each tetranucleosomal structural unit, ultimately resulting in the formation of the final helical structure of the 30-nm fiber (Fig. 4, panel A).

The structure of gH5 was solved earlier at 2.5 Å resolution by using multi-wavelength anomalous diffraction on crystals of the selenomethionyl protein [30]. However, despite several decades of effort, the structural basis of how H1 interacts with the nucleosome remains elusive. Recently, using solution nuclear magnetic resonance spectroscopy and other biophysical methods, Bai and colleagues have shown that the globular domain of Drosophila H1 forms a bridge between the nucleosome core and one 10-base pair linker DNA in an asymmetric manner. Its α3 helix faces the nucleosomal DNA near the dyad axis, which confirms earlier biochemical and biophysical evidence [44,45]. Interestingly, it was also shown that two short regions in the C-terminal tail of H1 and the C-terminal tail of one of the two H2A histones are also involved in the formation of the H1-nucleosome complex, which is consistent with previous observations [9,50]. Based on X-ray and NMR structures of gH5 and gH1, mutation analysis demonstrated that the globular domain of the linker histones contains two DNA –binding sites [51]. However, in our cryo-EM structure of the 30-nm fiber, the H1 directly interacts with both the dyad and the entering/exiting DNAs in a three-contact mode (Fig. 3, panel C), which is in agreement with previous suggestion [52]. In

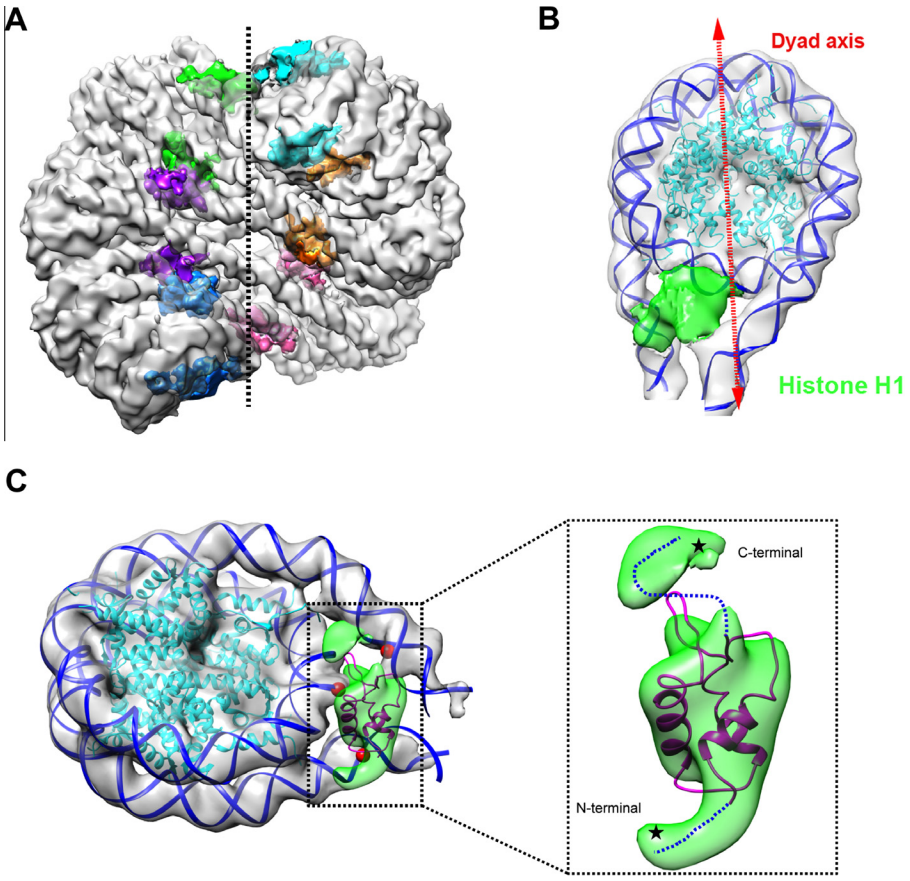

Fig. 3. Asymmetric location of histone H1 in the chromatosome and the 30-nm fiber. (A) The H1 linker histones, which are highlighted by different colors, locate inside 30-nm chromatin fibers. (B) Asymmetric location of histone H1 in the chromatosome. The putative histone H1 is highlighted in green. (C) Interaction between histone H1 and nucleosome core. The areas of three-contact interaction between H1 and the nucleosomal core are indicated with red dots. The presumptive H1 densities are magnified and fitted with the secondary structures of gH5. Adapted from [28].

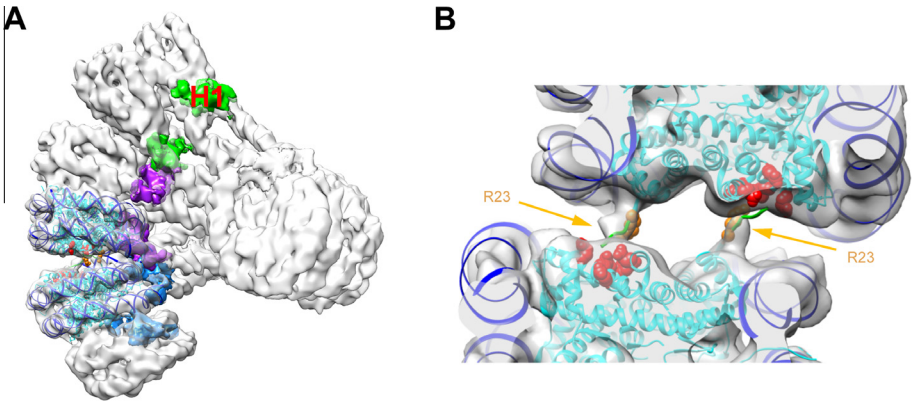

Fig. 4. Interactions between the tetranucleosomal units within the 30 nm fibers. (A) The asymmetric H1–H1 interactions between the tetranucleosomal units within 30 nm fibers. The locations of H1 are highlighted. (B) Interactions between the N-terminus of H4 (Yellow) and the acidic patch of adjacent H2A–H2B dimer (Red) at the interface between tetranucleosomal units. Adapted from [28].

addition, our structure also revealed that the extended N- and C-terminal domains of the linker histone H1 can interact with each of the linker DNAs (Fig. 3, panel C), which would be consistent with previous observations [37,42].

4. Nucleosome-nucleosome interactions within the 30-nm fiber

Nucleosome-nucleosome interactions have been observed as crystal packings in the structures of nucleosome cores [2] and histone octamers [53]. Among them, the interactions between the N-terminus of the H4 and the H2A-H2B acidic patch of a neighboring nucleosome [2] has been demonstrated to be directly relevant to the formation of chromatin fibers [22,54], which was not found, however, within the tetranucleosome crystal [24]. Instead, an interaction between the H2B-helix α1/αC and the H2A-helix α2 of neighboring octamers in each dinucleosomal stack was observed within the tetranucleosome crystal structure [24]. Because it is difficult to build a fiber model by only using this interface due to the steric clashes, an idealized model had to be constructed by using the proven H4 tail-acidic patch interaction [24]. Interestingly, our 3D cryo-EM structure reveals that both interaction modes play important roles in the folding of chromatin fiber, which is in a good agreement with previous predictions [2,24,55]. However, in contrast to previous assumptions of a sequential arrangement of nucleosomes with uniform orientations, we observed alternating internucleosomal contacts between spatially adjacent nucleosomes in our 3D cryo-EM structure of 30-nm fiber. This observation is supported by previous the dinucleosomal repeat patterns in the DNase I digestion [15,56]. In contrast to earlier predictions [2], the tetranucleosome emerges now as the main constitutive repeating unit of the fiber, which allows contact between the exposed surfaces of two H2A-H2B core histone dimers, similar to that observed previously in the tetranucleosome crystal structure (Fig. 2, panel D). However, no interaction was observed between the H4N-terminus and the acidic patch of the neighboring nucleosomes within the tetranucleosomal unit. Instead, between the tetranucleosomal units, there are contacts between the N-terminal tail of H4, primarily involving residue Arginine 23 and the acidic patches of the H2A–H2B dimer on the faces of the opposite nucleosomes in their respective adjacent units (Fig. 4, panel B), which also account for the twist between the tetranucleosomal units. To this end, it is of great interest to note that an 'arginine-anchor' binding to the H2A–H2B acidic patch, which plays an important role in the nucleosomal recognition, has been observed in all chromatin factor-NCP crystal structures [57]. In comparison to the closely stacked nucleosomes within the tetranucleosomal structural unit, the apparent gaps present between the tetranucleosomal units may provide a platform for histone modifications or other architectural proteins, allowing the modulation of the internucleosomal surface interactions required for the regulation of the 30-nm fiber structure. Our results also fully agree with recent cryo-electron tomography studies carried out in situ on chromatin fibers of chicken erythrocyte nuclei [58]. Using a chromatin model system containing up to four nucleosomes, Shogren-Knaak and colleague found that the ligated tetranucleosomal arrays undergo intra-array compaction in a histone H4 tail-independent manner [59], suggesting that the interaction between H4 tail and the acidic patch of adjacent nucleosomes is not responsible for the compaction of the tetranucleosomal unit. Interestingly, the cryo-EM structure of histone octamer helical tubes suggested that an internucleosomal twofold symmetric four-helix bundle, formed between pairs of H2B-α3 and H2B-αC helices of neighboring octamers, stabilizes the chromatin fiber [60]. However, the H2B-α3/αC internucleosomal four-helix bundle was not observed in our cryo-EM map of reconstituted chromatin fiber.

5. The polymorphisms of the 30-nm chromatin fiber and its epigenetic regulation

Although the precise details remain unknown, it is becoming clear that chromatin is of polymorphic nature, and that every state depends on both internal and external factors [3]. Deciphering the factors involved in chromatin folding is not only important for the understanding of DNA compaction, but also for the understanding of the mechanisms that regulate the accessibility to the primary DNA sequence of regulatory factors and complexes that modulate chromatin metabolism, such as DNA transcription, replication, repair and recombination [3,61].

The length of the linker DNA, as expressed in terms of the nucleosome repeat length (NRL), is one of the important internal factors known to affect the structure of the chromatin fiber [61]. In the native chromatin, the NRL is known to be highly variable, and the question arises as to what happens to the chromatin fiber when NRLs of differing sizes are assembled rather than being of constant length, as used in previous models. Crystal structures of nucleosome core particles indicate that the nucleosome core particle is very rigid, and that the ends of the 147 bp DNA are well defined. Therefore, an addition of 1 bp to the linker DNA changes the orientation of a nucleosome with respect to its adjacent neighbors by approximately 36°. Our knowledge as to whether NRLs of the fibers are discrete units or whether they change continuously is limited. This is in part due to the fact that micrococcal nuclease (MNase)

digests rarely provide a resolution higher than ±4 bp. Genomic studies of the preferred internucleosome separation lengths in several organisms have shown some partial preference for multiples of 10–10.6 bp, but also some pronounced intermediate lengths between 200 and 210 bp [62,63]. The NRL variability poses a major methodological problem, as it is extremely difficult to obtain crystallographic or high-resolution images on the basis of such heterogeneous chromatin. To circumvent the NRL variability problem, and to gain highly detailed structural information, computational efforts in modeling the structure of compact chromatin fibers have focused primarily on the role of electrostatic internucleosomal interactions, as well as the length and local geometry of linker DNA [64–66]. These studies showed that the twist-angle of the nucleosomes along the DNA plays an important role in determining the straight-linker superhelical structure. In particular, the nucleosomes prefer to be oriented with their symmetry axes parallel to the fiber axis for repeat lengths that are integer multiples of the DNA pitch, and with perpendicular axes for half-integer multiples of the pitch [66]. Extended structures are formed for repeat lengths close to an integer multiple of pitch, with much shorter fibers of near half-integer multiples [66]. In addition, some linker DNA lengths result in a collapse of the superhelix into a planar structure, but such structures would involve an overlap of the nucleosomes themselves and are therefore unphysical. In this case, the ground-state structure requires deformation of the linker DNA to reposition the nucleosomes so as to avoid steric overlap [66]. Most recently, the effect of a wide range of intrafiber NRL variations in chromatin fiber structure was explored through the Monte Carlo (MC) simulations of the mesoscale chromatin model [67]. The authors found a remarkable effect of non-uniform NRLs in the organization and compactions of chromatin fiber, with a wide range of different architectures emerging (highly bent narrow forms, canonical and irregular zig–zag fibers, as well as polymorphic conformations), depending on the NRLs mixed together [67]. In this study, three different simulations were performed, and they found that non-uniform short linker DNA fibers exhibited a compact conformation, with a large bend along the fiber axis. Using medium- to long-range linker DNA and moderate NRL variation, heteromorphic conformations with both straight (zig–zag type) and bent linker DNAs were observed, which was consistent with the previous finding from EM-assisted nucleosome interaction capture (EMANIC) analysis that suggested the two-start zig–zag conformation and one-start solenoid structure with bent linker DNAs co-existed in a structurally heteromorphic chromatin fibers with a NRL of 207 bp [25]. However, when the NRL variations were large, the resulting fibers were extremely polymorphic, including zig–zag structures, bent fibers, hairpin-like conformations and loops.

The elasticity of the linker DNA allows for dozens of distinct candidate structures of a compact chromatin fiber. Indeed, electron microscopy measurements revealed that a short NRL (167 bp) results in the formation of narrow fibers (21-nm diameter) that display a clear zig–zag topology, whereas a medium NRL (197 bp) forms highly compact 30-nm interdigitated solenoid structures [23], which was further supported by single-molecule stretching analysis of chromatin fibers with NRLs of 167- and 197-bp [68]. Our own cryo-EM structure revealed that the chromatin fibers with NRLs of 177 and 187 bp forms two-start left-handed helical fibers, with diameters of 27–30 nm and a clear zig–zag conformation [28]. However, recent experiments have shown that small NRLs deviations (±2 and ±4 bp from the mean repeat) do not change significantly the folding and compaction of chromatin [23,69]. This shows that the intrinsic structure of the fiber must allow for several different repeat lengths (differing not only by 10n bp, where n is an integer) and accommodate a non-integer periodicity of the DNA helix (10.5 bp/turn). To rotate the nucleosomes away from their preferred angle to the axis, a high amount of energy would be required. The feasibility of any structure is expected to depend on the geometry of the DNA attachment to the nucleosomes. Differences in fiber structure due to changes in the entry/exit geometry may also arise in vivo through partial unwrapping of the nucleosomes, either by acetylation of histone tails [70], the introduction of histone variants such as CENP-A [71] and H2A.Bbd [72], or through the action of nucleosome remodeling complexes [73]. Drastic alternation of nucleosome geometry might also occur if nucleosomes dissociate into hemisomes containing one copy of each histone protein, as reported for Drosophila centromeric nucleosomes [74]. In addition, EM experiments of reconstituted nucleosomes showed that a short stem was observed on the linker DNA of nucleosome [8,37]. At present, it is not clear whether the size of the stem is constant or whether it depends on the NRL and the type of the bound linker histone. It has been shown that the globular domain of H1 plays important roles in maintaining the orientation of nucleosome discs relative to the fiber axis [75]. Thus, geometrical considerations for the fiber should allow for some differences between the actual linker lengths and the real distances between consecutive nucleosomes. In addition, variegation effect studies in Drosophila have shown that gene silencing is accompanied by higher compaction, with nucleosomes being spaced more regularly [76]. Together, these results suggest that the nucleosome repeat lengths in vivo could also be defined by existing structural constrains such as nucleosome–nucleosome interactions and the binding of linker histone H1.

Except for the variation in NRLs, the chromatin fiber could also be regulated by chromatin remodeling factors, histone modifications and replacements, linker histone H1 removal, and changes in the non-histone protein complement [3]. As discussed above, the H1–H1 interaction and H4 tails-the acidic patch interaction between tetranucleosomal units have been shown to play critical roles in the formation of the twisted helix of 30-nm chromatin fiber. Interestingly, it is to note that the apparent gaps formed between tetranucleosomal units in our 3D cryo-EM structure may provide a platform for epigenetic regulation of chromatin fiber structures, either by histone modifications/replacement or through recruitment of other architectural proteins through altering the inter-nucleosomal surface interactions.

Acetylation of core histone tails is perhaps one of the best-characterized posttranslational modifications known to modulate both chromatin structure and gene activity, as modifications serve to specifically recruit transcription factors and other activities to the chromatin, or by modifying tail interactions and thus chromatin structure in a more direct manner [3,77]. Early experiments indicated that acetylation directly alters interactions of histones with DNA and/or protein in arrays to destabilize the chromatin structure and facilitate transcription [78]. More recent studies provided evidence that the effects of acetylations of histones on transcription appears to be primary at the level of chromatin folding, as acetylation does not significantly increase transcription through individual nucleosomes [79]. Similarly, acetylation of histone was found to decrease the overall stability of mononucleosomes only marginally [80], but was shown to impair strongly the folding of oligonucleosome arrays into higher-order structures [10,81,82]. A threshold effect of acetylation was found in the disruption of higher-order chromatin structure, however, disruption of the folding of chromatin fiber was also observed with acetylation mimics located only on the H4 tail domain, and acetylation of H4K16 was shown to reduce $MgCl_2$-dependent array folding as much as acetylation at lysines 5,8,12, and 16 within the H4 tail [83–85]. Using in vitro reconstituted nucleosome arrays, it has previously been shown that in the absence of linker histone H1, acetylation of H4K16 results in a major unfolding of the reconstituted

nucleosome arrays [85]. Interestingly, this effect may be due to specific cation binding to the pocket within the H2A acidic patch, thought to be occupied by H4K16 in condensed chromatin structures [83]. However, the molecular mechanisms involved in this process, as well as the precise details of the structure still remain unknown, more importantly, the potential role played by linker histones in this situation remains to be solved. Indeed, the effects of core histone acetylation on chromatin fiber folding in the presence of linker histone H1, seem to be minor [86] when compared to the unfolding that is observed in its absence [81]. In contrast, Robinson et al. [84] demonstrated that the histone H4 tail is essential for the compaction of nucleosome arrays into the 30 nm chromatin fiber. However, partial acetylation of K16 in the N-terminal tail of histone H4 strongly inhibits the formation of the 30 nm chromatin fiber to a greater degree than deletion of the H4N-terminal tail in the presence of a linker histone.

The acidic patch on the surface of H2A and H2B was also shown to be involved in the formation of the 30-nm fiber through interacting with H4 tails of adjacent nucleosome. Therefore, incorporation of non-allelic variants of the major core histone proteins, which can change the acidic patch, may also alter the folding and stability of higher-order chromatin structures [3]. For example, the H2A variant H2A.Z possesses a patch that is more acidic than that of canonical H2A histone, due to the replacement of asparagine and lysine residues in the patch with aspartic acid and serine residues, respectively [87]. This intrinsic property of H2A.Z possibly contributes to the formation of more compact secondary structures than observed with arrays containing H2A [88,89], a property lost when the relevant residues are mutated back to that of H2A. Interestingly, the more acidic patch of H2A.Z also inhibits array self-association into tertiary structures, suggesting competing functions for the H4 tail domain. In contrast, the acidic patch of the H2A variant H2A.Bbd, which is associated with more actively transcribed regions of chromatin, is less acidic than canonical H2A and results in a less stably folded secondary structure that is more transcriptionally competent than that of H2A arrays [90]. In addition, the C-terminal tail of H2A has been shown to play an important role in the binding of the linker histone H1/H5 with nucleosome cores, with the C-terminal tail of histone variant H2A.Z disfavoring H1 binding [50,65]. Therefore, H2A.Z also modulates the folding of chromatin fiber by weakening the binding of linker histone H1 [91]. Interestingly, we have also demonstrated that the incorporation of histone variant H3.3 can impair the compaction of chromatin fiber and counteract H2A.Z-mediated chromatin compaction through an unknown mechanism [89]. The C-terminal tail of H2A can also contact nucleosomal DNA near the center of the nucleosome core, but "shifts" to contact DNA near the edge of the core region in nucleosomes and oligonucleosomal structures containing the linker DNA [92]. Therefore, it would be interesting to study whether the modification of C-terminal tail of histone H2A, such as ubiquitination of H2AK119, can modulate the folding of the chromatin fiber. In addition, H1–H1 interaction (most likely through its global domains) was also found to be important for stabilizing the 30-nm chromatin fiber, thus the modifications of global domains of H1/H5 could provide a new mechanism for the epigenetic regulation of the compaction of the chromatin fiber. Indeed, the citrullination of Arg54, which results in the loss of a positive charge and a gain in hydrogen-bonding ability, can impair the compaction of chromatin fiber by reducing the binding of H1 to nucleosome, and probably also lowering the strength of interaction between H1–H1 [93]. In addition, phosphorylation of the carboxy-terminal domain (CTD) of histone H1 has been shown to be involved in metaphase chromatin condensation and in interphase chromatin relaxation through charge neutralization [94] and effects on secondary structure of CTD [95]. Similarly, phosphorylation at Ser27 in the N-terminal tail of histone H1 was shown to regulate the mobility and binding of H1 to condensed mitotic chromatin [96]. Of note, higher eukaryotes contain a number of linker histone variants (about 11) that possess C-terminal tails that diverge considerably in their primary AA sequence [31]. The functions of linker histone H1 and its isoforms in the formation of higher-order chromatin structure and gene regulation have been extensively discussed elsewhere [31,32]. However, how the sequence variations of linker histones (e.g. the two species of H1 and H5 in chicken erythrocytes), or the posttranslational modifications of the H1 influence the stability of the higher-order structure as well as the accessibility of the linker DNA still remains largely unknown.

The acidic patch was shown recently to provide binding sites for several non-histone proteins, many of which are thought to regulate chromatin structure [55]. These include the chromatin architectural factor HMGN2, which binds to nucleosome cores and generates a more open and transcriptionally permissible chromatin secondary structure. HMGN2 has recently been shown to interact directly with the acidic patch within nucleosome cores [97]. Likewise, HP1, a heterochromatin-associated protein functioning in the formation of highly compact heterochromatin, is thought to interact with the acidic patch, and prefers to bind H2A.Z-containing nucleosomal arrays [90]. These proteins compete in part with the H4 tail domain for binding to the acidic patch and alter chromatin folding and self-association. The herpes virus latency-associated nuclear antigen (LANA) helps to tether the viral genome to the host genome by binding to the acidic patch with high affinity [98]. Notably, a peptide from the LANA protein containing only the interacting region competes with the H4 tail for binding to the patch and results in an increased propensity for salt-dependent chromatin folding and oligomerization [99]. Additional evidence provided by these investigators indicates that association of LANA (or the H4 tail) with the patch neutralizes repulsive forces between nucleosomes, thereby stabilizing secondary structures. Interestingly, peptide-dependent displacement of the H4 tail from the acidic pocket prevents the intra-array compaction, but stimulates tertiary structure formation, perhaps by making more H4 tail domains available for inter-array contacts [99]. Finally, several other chromatin-associated proteins contact the nucleosome partially through the H2A/H2B acidic patch, suggesting that this may be a general mode of interaction [100–102].

Another class of nucleosome-associated interactions that affect higher-order chromatin structure is mediated by non-histone chromatin-associated proteins [3]. Therefore, there must exist alternative secondary structures of condensed chromatin, with or without H1 histone, resulting from the interaction with non-histone "architectural" factors and other trans-acting factors, such as HMGNs, PcG proteins, MENT, $MeCP_2$, MBT proteins and HP1 [3]. Several chromatin-associated proteins have shown to play a direct role in the formation and/or maintenance of repressive chromatin fiber, including the polycomb group protein PRC1 and PRC2 [103,104], MENT [105], MeCP2 [106]. On the other hand, both malignant-brain-tumor (MBT) proteins [107] and heterochromatin protein 1 (HP1) [108,109], which can specifically recognize and bind to methylated histone tails, compact nucleosomal arrays in a manner dependent on histone methylations. It would be interesting to perform structural analysis of reconstituted chromatin complexes consisting of H3K9 and H4K20 methylation heterochromatin markers and interacting proteins, such as HP1 and MBT proteins [61]. Recently, it was shown that homodimerization is required for the binding of both Rhina (a homolog of HP1 in Drosophila) and Swi6 (*Schizosaccharomyces pombe* HP1 homolog) proteins to chromatin whose histones contain H3K9me3 marks [110]. Moreover, the recognition of H3K9-methylated chromatin by Swi6 in vitro also relies on an interface between two chromodomains (CDs), which results in tetramerization of Swi6 on the

surface of the nucleosome, generating two vacant CD sticky ends in the process [109]. Note that HP1 has been shown to interact with the acidic patches of nucleosomes, thus the binding of HP1 may also modulate chromatin compaction by interferring with the H4 tails-the acidic patch interaction between tetranucleosomal units. Therefore, it is speculated that HP1 proteins stabilize the 30-nm chromatin fiber via bridging the adjacent methylated nucleosomes between tetranucleosomal units. Recently, Huang and colleagues showed that two histone H3K9me3 peptides bind to the Rhi-CD dimer in an anti-parallel way in their crystal structure study. Based on our recent 3D cryo-EM structure of 30-nm chromatin fiber, they proposed that Rhi protein stabilizes the chromatin fiber via bridging the stacked nucleosomes on both strands of the double helix of chromatin fiber [110]. Similarly, MBT proteins and the condensin II complex have been shown to condense chromatin through binding to the monomethylated lysine 20 of histone H4 tails [107,111]. Therefore, MBT proteins and condensin II complex may also play an important role in regulating the formation of chromatin fiber via interfering with the interactions between H4 tails and the acidic patches present between tetranucleosomal units during mitosis.

6. Organization of the chromatin fiber within the nucleus

The 30-nm fiber has long been thought to be the first level of the hierarchical chromatin compaction pathway, but the existence of the 30-nm fiber in vivo still remains very controversial, because high-resolution imaging of chromatin in living cells had not been possible until now [112,113]. The initial identity of the 30-nm fiber in situ came from cryogenic electron microscopy (Cryo-EM) studies, undertaken in echinoderm sperm and nucleated chicken erythrocytes chromatin [114,115]. Further evidence for the existence of a 30-nm fiber came from earlier attempts to biophysically characterize the isolated native chromatin fiber from different biological systems [11].

Previous cryo-EM observations of starfish spermatozoids and isolated chicken erythrocyte nuclei revealed that under low-salt conditions, an open irregular zig–zag conformation exists, with an increase in salt concentration resulting in progressive compaction of the chromatin fiber [116]. One earlier study also provided strong evidence for the presence of a 30-nm fiber structure, even after high-pressure freezing and thin section of vitreous material [114]. Most recently, using cryoelectron tomography of vitreous sections of chicken erythrocyte chromatin in nuclei, Frangakis and colleagues showed that the most predominant form of chromatin in chicken erythrocyte nuclei is indeed a 30-nm fiber. Importantly, these fibers were observed to be arranged in a two-start helix formation, with approximately 6.7 nucleosomes per turn, in which the nucleosomes are juxtaposed face-to-face. The stacked nucleosomes were shown to be shifted off their superhelical axes, with an axial translation of approximately 3.4 nm and an azimuthal rotation of approximately 54° [58]. Intriguingly, this geometry strongly resembles the structure of the tetranucleosome [24], and also resembles our 3D cryo-EM structure, which revealed a 30-nm fiber composed of a left-handed two-strand double helix consisting of tetranucleosomal units [28]. Furthermore, electron tomograms of plunge-frozen isolated chromatin from chicken erythrocyte in both open and compacted forms were recorded. Frangakis and colleagues found that in compact chromatin, the nucleosomes are arranged in a predominant face-to-face stacking (mainly as doublet stacks) organization [58,117]. Although the path of the DNA cannot be observed directly under such compact conditions, it is evident that the nucleosome stacks form a "double track" conformation, which was confirmed by our recent 3D cryo-EM structure of 30-nm fiber [28]. The in vivo existence of face-to-face nucleosome stacking is also supported by a peak in the 5.5–6.2 nm range, as observed previously in the X-ray scattering profile of interphase nuclei and metaphase chromosomes [118]. Together, this body of experimental evidence strongly suggests that stacking interactions of nucleosomes are an important mechanism for generating chromatin compaction both in vitro and in vivo.

As discussed above, the NRLs play an important role in the organization of the chromatin fiber. To determine the nucleosome-nucleosome interaction in chromatin with a longer NRL, the chromatin isolated from starfish (*Patiria miniata*) spermatozoids, which contains exceptionally long NRL of 222 bp, was analyzed. It has been hypothesized that the chromatin fibers with longer NRL adopt a different conformation from that of fibers containing short NRLs [16]. This study showed that the predominant form of chromatin compaction in starfish spermatozoids is also face-to-face stacking, with the nucleosomes organized in a "double-track" conformation, even though the overall fiber structure was different to the compact chicken erythrocyte chromatin [117]. However, the larger azimuthal rotation in the fiber results in a more homogeneous nucleosome distribution when compared to the idealized cross-linker model, where stacked nucleosomes form two dense gyres that are widely separated in both cases. Close face-to-face nucleosome stackings have previously been demonstrated in various crystal structures [2,24]. In the contrast, a center-to-center distance of stacked nucleosomes in situ is approximately 10.3 nm, leaving a large distance of at least 3 nm between the two octamer cores, which is much larger than that reported in crystal structures [58]. This binding distance excludes core-to-core interactions but allows interactions mediated by histone N-terminal tails in a tail-to-core interaction. It is of great interest to note that different face-to-face juxtaposition of nucleosomes was observed in our 3D cryo-EM structure of 30-nm fiber [28]. The center-to-center distance between the nucleosome stacks within tetranucleosomal unit is close enough to allow the core-to-core interactions mediated by H2A/H2B four helix bundles, however, a much larger gap was observed between the tetranucleosomal unit in our structure of 30-nm chromatin fiber and it excludes the above mentioned core-to-core interactions, but allows interactions mediated by H4 tails-acidic patch on the neighboring nucleosomes [28]. In addition to the chicken erythrocyte and starfish spermatozoid, a recent study showed that the facultative heterochromatin on the mouse rod photoreceptor cell also contains a 30-nm fiber organization [119].

DNase I has long been used to probe the higher-order chromatin structure in whole nuclei. Early DNase I analysis suggested that the linker DNA and linker histone is buried deep inside the 30-nm fiber, which is in agreement with previously published reports using other techniques [120–122]. In addition, digestion of whole nuclei with DNase I or DNase II produces a series of DNA fragments, the length of which is a multiple of the duplicate length of the nucleosome DNA repeat in nuclei of various origins (Avian erythrocytes and sperm of sea urchin) [123–125]. This unusual dinucleosomal periodicity of DNase I digestion pattern implies the existence of a particular structural unit that consists of at least 2 nucleosomes, which is to some extent in agreement with our 3D cryo-EM 30-nm fiber structure, where tetranucleosomes are found to be the repeating structural unit [28]. Interestingly, dinucleosomal symmetry of the higher-order chromatin structure has been shown previously to be independent of nucleosome repeat length [56,123,124]. With the recent development of deep sequencing techniques, it is now possible to map the location of the structural units (comprised of dinucleosomes or tetranucleosomes) across the whole genome. In addition, DNase I digestion also results in an asymmetrical protection of nucleosomes in nuclei, which suggests that the vast majority of nucleosomes has an alternating orientation and asymmetrical organization in vivo [56,123–125]. The

alternating orientations of nucleosomes in the fiber may result from criss-crossing linkers. Intriguingly, our 3D cryo-EM analysis indicates the presence of a twisted helical fiber structure containing alternately tilted nucleosomes [28], which would provide an explanation for the observed DNase results. In contrast to artificial arrays, which are of defined nucleosome repeat length and uniform histone composition, native chromatin has inherent local variations in nucleosome repeat length, modifications and variants of the core and linker histones. Despite this variability, native chromatin shows remarkable structural regularity, as observed in the reconstituted compact 30-nm fibers in vitro.

Despite this overwhelming body of evidence, few reports exist for the presence of the 30-nm fiber within intact cells of any other organisms as discussed previously [126]. Even in the highly compacted heterochromatin regions of the nuclei of human and mouse cells, 30-nm chromatin fibers were never observed, even when investigated with the highly sophisticated EM techniques currently available [112,113,127,128]. Although they used a powerful combination of cryo-electron microscopy and image processing, Dubochet and colleagues could not find any regular 30-nm chromatin fibers within mitotic chromosomes in situ [112]. Also, recent studies using SAXS (Small angle X-ray scattering) analysis combined with computational modeling were unable to detect regular structural features that were larger than 11 nm in the interphase chromatin and mitotic chromosomes [128,129]. As discussed above, phosphorylations of histone H1 and monomethylation of histone H4 lysine 20 have been shown to regulate the formation of 30-nm chromatin fiber through different mechanisms [94–96,107,111]. Interestingly, both phosphorylations of H1 and monomethylation of H4 lysine 20 were found to be peak in M phase [96,111], therefore, it is reasonable to speculate that the 30-nm chromatin fiber may be disrupted in mitotic chromosomes by these modifications on histone H1 and histone H4 tails. In addition, using cryo-EM tomography to study the picoplankton *Ostreococcus tauri*, Gan et al. found that *O. tauri* chromatin resembles a disordered assembly of nucleosomes, without any indication of the presence of structures resembling the 30-nm chromatin fiber [130]. Using another EM-based imaging method called electron-spectroscopic imaging (ESI), Bazett-Jones et al. found that pluripotent mouse cells contain highly dispersed meshes of 10-nm fibers. Again, no 30-nm fibers were found in the various types of mouse cells, even in the condensed domains of heterochromatin regions [127,131,132]. Thus, strong evidence for the presence of ordered hierarchical folding (as a general mechanism for chromatin compaction) across the different model systems remains conspicuously absent.

7. Conclusions and perspectives

Our 3D cryo-EM structure of 30-nm chromatin fiber provides an excellent starting point for the elucidation of the fundamental structural aspects of the elusive 30-nm chromatin fiber. However, due to the relative low resolution of approximate 11 Å, the precise details about the various interactions, either between nucleosome stacks within tetranucleosomal units or between H1–H1 of different units, remain to be elucidated. With recent advances in direct electron detection and image processing, the resolution of cryo-EM analysis is now beginning to rival X-ray crystallography [133]. Thus, the resolving of the structure of 30-nm chromatin fiber at a higher resolution by using the new electron detector and imaging processing is now imminent. Such high-resolution structure will provide much-needed structural details for a better understanding of the interactions between nucleosome–nucleosome as well as H1–H1. This will undoubtedly advance our insights into the functions of the tails of both core histone and linker histone H1 in the formation of 30-nm chromatin fiber. In addition, our 3D cryo-EM structure of the 30-nm fiber implies that the large gap present at the interface between tetranucleosomal units, which is mediated by the interactions of H4 tails and acidic patches on neighboring nucleosome, provide an excellent platform for epigenetic factors/mechanisms to modulate the folding of the chromatin fiber. It would be of great interest to determine the high-resolution cryo-EM structure of reconstituted chromatin fiber containing histone variants or histones carrying different histone modifications. As discussed above, the C-terminus of H2A has important functions in the formation of higher-order chromatin structure, thus it will also be of interest to investigate whether the histone modifications on histone H2A C-tail (such as monoubiquitination of histone H2A at lysine119) and histone H2A variants with different C-tail (such as macroH2A and H2A.X) can affect the compaction of chromatin fiber. Regarding the variation of NRL in vivo, the reconstitutes with a combination of different NRLs will be also a good candidate for cryo-EM study in the future. These further studies will not only enhance our understanding of the functions of histone modifications and variants in epigenetic regulation of chromatin compaction, but also provide an opportunity to find novel structural features within the 30-nm chromatin fiber. Crucially, some chromatin modifications may have no direct effect on the structure of chromatin fiber, but they might instead modulate the chromatin structure indirectly, presumably by recruiting chromatin interacting factors apart from linker histones such as HP1, MeCP2 and MBT proteins. Deciphering the structural role of these additional chromatin binding proteins in different chromatin contexts (i.e. different modifications) would also be of tremendous importance. Recently, single-molecule approaches have been applied to investigate the dynamics of chromatin fiber folding [68,134], thus it would be interesting to study the force and the potential epigenetic regulations involved in different nucleosome-nucleosome interactions (intra- or inter-tetranucleosomal units) during the step-wise folding and unfolding of the 30-nm chromatin fiber.

Another important question is how much the structural results from the in vitro studies mirror the structure of the actual "native" 30-nm chromatin fiber? To solve this puzzle, the study of the organization of chromatin will require the development of advanced techniques. Previously, cryo-EM studies in mammalian cells revealed no existence of regular 30-nm chromatin fibers in mitotic and interphase chromosomes in human and mouse cells [112]. Cryo-EM can examine biological sample in close to their native states, however, it is limited by the fact that only a small portion of the sample can be detected [112]. However, the recent development of coherent X-ray diffraction imaging (CXDI) has made it possible to visualize a single chromosome using X-rays [129]. In addition, X-ray free electron lasers (XFELs) have been developed that produce high peak-brilliance coherent hard X-rays. Potentially, a combination of CXDI and XFELs will allow the visualization of chromosomes at a resolution within the nanometer range. To visualize and quantify the ultrastructure of cryopreserved cells, two other combinatorial methods, the combination of Soft X-ray tomography (SXT) with cryogenic confocal fluorescence tomography (CFT) [135] and the combination of cryo-EM with super resolution fluorescence imaging techniques, have been developed recently. It is to be expected that by the application of these combinational imaging techniques in the future, more structural details for the organization of chromatin fiber can be obtained in situ.

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